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Expansion of Space Station Diagnostic Capability to  
Include Serological Identification of Viral and  
Bacterial Infections

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EXPANSION OF SPACE STATION DIAGNOSTIC CAPABILITY TO  
INCLUDE SEROLOGICAL IDENTIFICATION OF VIRAL AND  
BACTERIAL INFECTIONS

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It is necessary that an adequate microbiology capability be provided as part of the Health Maintenance Facility (HMF) to support expected microbial disease events during long periods of space flight. The applications of morphological and biochemical studies to confirm the presence of certain bacterial and fungal disease agents are currently available and under consideration. This confirmation would be greatly facilitated through employment of serological methods to aid in the identification for not only bacterial and fungal agents, but viruses as well. A number of serological approaches were considered, particularly the use of Enzyme-Linked Immunosorbent Assays (ELISAs), which could be utilized during space flight conditions.

A solid phase, membrane supported ELISA for the detection of Bordetella pertussis was developed to show a potential model system that would meet the HMF requirements and specifications for the future space station. A second model system for the detection of Legionella pneumophila, an expected bacterial disease agent, is currently under investigation. These preliminary studies demonstrate the capability of ELISA systems for identification of expected microbial disease agents as part of the HMF.

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## INTRODUCTION

The health and well being of individuals aboard a space station and possibly during future long space missions is of priority and must be assured. Certain expected clinical syndromes and diseases have been identified through an infectious disease conference conducted during October, 1985. Previous spaceflight studies indicate a high probability of cross-contamination among crewmembers during long confinements, such as the 90 day missions planned for the space station (12). Continual habitation, crowded conditions, possible immunosuppression, and other factors may create critical situations aboard the space craft. If a microbial disease is suspected, the major effort would be directed toward obtaining some indication of the specific kind of microorganism causing the problem. The exact nature of the etiologic agent would determine the severity of the disease, treatment, prophylaxis, and subsequent health measures for the space station environment.

The diagnosis of a microbial disease rests upon one or a combination of clinical signs and symptoms, morphological and biochemical identification of isolates, and/or serological procedures. Special procedures such as cell culture may also be required. One problem with limiting the scope of diagnosis to clinical signs and symptoms is that a particular microbe can sometimes produce infection having very different clinical characteristics and occurring in widely different areas of the body. For example, antibiotic-resistant Staphylococcus aureus may produce skin and subcutaneous tissue lesions as well as pneumonia, osteomyelitis, bacteremia, and acute membranous enterocolitis,

depending upon the means by which the organism gained entrance to the body, host resistance, antibiotic therapy, and other factors.

While the principle of one microorganism causing one clinical disease is often valid, there are many situations where this is not true. Indeed, pneumonias that are hardly separable clinically may be produced by several different kinds of bacteria and viruses. Correct diagnosis and treatment therefore heavily depend upon the abilities of the clinical laboratory.

It is important to note that serological procedures will not immediately take the place of needed morphological and biochemical identification of bacteria or fungi; however, they are frequently used to verify, compare, and further substantiate those results as well as provide a means to directly identify viruses or the immunological response to a viral infection.

Over the past few years, many new immunological methods have been developed which now provide the clinical laboratory with a large array of potentially valuable diagnostic tools. Antibodies and antigens labeled with radioisotopes or fluorescent dyes, or affixed to particulate materials, have been used extensively for immuno-diagnosis over the past three decades. These methods do have disadvantages. Immunofluorescence, for example, usually depends upon a subjective assessment of end result, and the technique is frequently laborious. Radioimmunoassay requires expensive equipment and carries the risk of radioactive exposure and contamination.

The concepts that antigen and antibody can be attached to a solid

phase support yet retain immunological activity, and that either can be coupled to an enzyme and the complex retain both immunological and enzymatic activity, led to the development of Enzyme-Linked Immunosorbent Assays (ELISAs). Antibodies and antigens have been shown to readily attach to plastic surfaces (such as polyvinyl or polystyrene) either by passive absorption or chemical conjugation, and still retain immunological activity. Antibodies and antigens have been linked to a variety of enzymes including glucose oxidase, peroxidase, and alkaline phosphatase. The positive factors for ELISAs include low cost, reagent stability, safety, sensitivity, reproducibility, and ease of procedure. The procedures are simple enough to be performed in even poorly equipped laboratories.

It appears likely that the space station diagnostic capability will require immunological testing applicable to the identification of microorganisms, particularly viruses. In recent years, there has been increasing emphasis on accurate, reliable, and quick immunological procedures for the identification of many microorganisms and/or the immunological responses of the host toward infection. Most current procedures have been developed for use in clinical laboratories and not designed for a space station environment. It appears and is reasonable that a number of some existing procedures, particularly solid phase immunoassays, could be modified in regard to uniformity and standardization for use aboard the space station. This project was designed to illustrate the concept of a solid phase, membrane supported ELISA to demonstrate the capability of ELISA systems for

identification of expected microbial disease agents aboard the space station.

The main purpose of this project was to assess the current ELISA technological trends and procedures in the immunological identification of viral and bacterial diseases, particularly those microorganisms expected to cause illness aboard a space station, and to determine which procedures could be effectively implemented into the space station microbiology diagnostic capability as part of the HMF (Health Maintenance Facility).

## MATERIALS

Equipment. A 96 well Bio-Dot filtration apparatus (#170-6550) was obtained from Bio-Rad Laboratories, Richmond, Ca. 94801.

Buffers. A 20 mM Tris buffered saline (TBS), pH 7.5, was prepared by adding 4.84 g Tris (Bio-Rad) to 58.48 g NaCl, brought to a 2.0 l volume with deionized water. The buffer was adjusted to pH 7.5 with HCl.

Blocking Solution. A 3.0% BSA-TBS solution was prepared by adding 3.0 g of bovine serum albumin (Difco) to 100 ml of TBS.

Wash Solution. A wash solution containing 0.05% Tween-20 was prepared by adding 0.5 ml of Tween-20 (Bio-Rad) to 1 l of TBS.

Antibodies. A human serum pool containing antibodies to Bordetella pertussis was obtained from the clinical laboratories at NASA, Johnson Space Center. Antiserum to Legionella pneumophila (serogroup 1) was obtained from Dr. Hazel Wilkinson, the Department of Health and Human Services, Center for Disease Control, Atlanta, Ga. 30333. Horseradish peroxidase conjugated (HPR) goat anti-rabbit (#170-6500) and (HPR) goat anti-human (#172-1050) were obtained from Bio-Rad Laboratories.

Antigens. Bordetella pertussis antigen (#2515) was obtained from Difco Laboratories, Detroit, Mi. 48232. This concentration of this preparation was 2 x McFarlands units (equivalent to approximately  $1.8 \times 10^9$  organisms/ml). Legionella pneumophila antigen was prepared from a ATCC 3152 (serogroup 1) lyophilized culture vial (13). The ATCC vial was broken and the lyophilized material was dissolved into

4 ml of Trypticase Soy Broth. Four 15 x 100 mm plates containing 25 ml of buffered charcoal yeast extract (BCYE) agar was each inoculated with 1.0 ml of the dissolved material. The plates were enclosed in a plastic container to prevent the agar from drying out and were incubated at 35 C for 48 hr. The cells were suspended from each agar surface in 3.0 ml of sterile 0.01 M phosphate buffered saline, pH 7.2, with a pasteur pipette into a 25 ml sterile conical tube. The conical tube containing the cell suspension was boiled for 1 hr to kill the cells. The killed cell suspension was centrifuged at 1600 x g for 30 min, the supernatant discarded, and the cells resuspended in 2.0 ml of 0.01 M phosphate buffer, pH 7.2 for each 0.1 ml of packed cells. One drop of a 1:1000 methiolate solution was added for each 2.0 ml of preparation. The stock solution was stored at 4 C for 10 days to allow for the release of soluble antigen from the cells. The suspension was centrifuged at 1600 x g and the supernatant used for assay development.

Stock Chromogenic Substrate Stain Solution. Two substrates were utilized for comparison. O-phenylenediamine (OPD, Abbott Laboratories) was prepared by dissolving 12.8 mg into 5 ml of citrate phosphate buffer, pH 7.2, containing 0.02% hydrogen peroxide. 4-chloro-1-naphthol (4ClN, Bio-Rad) was prepared by dissolving 60 mg of 4ClN into 20 ml of ice cold methanol. Immediately prior to use, 0.06 ml of ice cold 30% hydrogen peroxide was added to 100 ml of room temperature TBS. The two solutions were mixed just prior to use.



## METHODS

The ELISA systems for both Bordetella pertussis and Legionella pneumophila utilized the Bio-Dot apparatus with the mounted nitro-cellulose paper. The procedure for assembly of the apparatus and preparation of the nitrocellulose paper was provided by Bio-Rad Laboratories (2). Briefly, the nitrocellulose paper was first soaked in TBS to ensure uniform protein binding and low background absorption. The cleaned and dried Bio-Dot apparatus was assembled, and the nitro-cellulose paper sheet wetted prior to being placed in the apparatus. The apparatus was appropriately tightened to insure that cross well contamination would not occur.

The flow valve was adjusted to allow the vacuum chamber to be exposed to the atmosphere and the appropriate wells to receive the antigen preparation were inoculated with a 0.05 ml volume. (Proteins bound were minute quantities of either antigen or capture antibody applied as a 0.05 ml volume of a concentration of 0.1-1.0 mg/ml.)<sup>2</sup> Nitrocellulose paper has a protein binding capacity of 0.08-0.1 mg/cm<sup>2</sup>. The entire sample was allowed to filter through the membrane by gravity flow (approximately 30 min). Each well was filled with the same volume of sample solution to insure homogeneous filtration of all sample wells.

After the antigen samples completely drained from the apparatus, 0.2 ml of a 3.0% BSA/TBS blocking solution was applied to each well. Gravity filtration was allowed to occur until the blocking solution completely drained from each well (approximately 30 min).

The flow valve was adjusted to vacuum and 0.4 ml of wash solution (TBS with 0.05% tween) was added to each well. The wash solution was allowed to completely drain from all wells. This process was repeated.

Following the wash step, the flow valve was opened to the atmosphere and 0.1 ml of the first antibody solution was added to each of the wells. The solution was allowed to completely drain from the wells, and another wash step performed.

With the vacuum off and the flow valve to the atmosphere, 0.1 ml of second antibody (HPR antibody against the first antibody) was added to each well. The solution was allowed to completely drain from the wells.

Following the second antibody step, the vacuum was turned on and a wash step performed. Immediately, 0.2 ml of a color development solution, either OPD or 4ClN was applied to each well. A positive ELISA reaction will be shown as color development depending upon the substrate utilized.

## RESULTS

Non-specific protein binding: A 2.0 cm disc of nitrocellulose paper was appropriately mounted in a modified millipore apparatus. The nitrocellulose was washed twice with TBS. A 2.0 ml aliquot of a BSA solution was allowed to pass through the membrane. A spectrophotometric reading (320 nm) for protein in the solution was taken before and after the solution passed through the membrane. The readings were compared, and it was calculated that the nitrocellulose paper retained approximately  $0.110 \text{ mg/cm}^2$  of total protein. This was corrected for the amount of protein released by a wash step.

Enzyme-substrate system: The indicator substrates, OPD and 4ClN, were tested for their interaction to HPR goat anti-human antibody attached to the nitrocellulose paper. Eight rows of 12 cells in the Bio-Dot apparatus were prepared identically, initially washed with TBS, followed by the application of 0.05 ml of serial dilutions ranging from 1:100 to 1:10,000 of the HPR goat anti-human serum. Each cell was blocked against additional protein binding by the coating buffer and washed with TBS. Different volumes of OPD substrate ranging from 0.05 to 0.3 ml, but consistent for each row was applied to the first four rows of the cells. Identical volumes were applied to the last four rows using the 4ClN. Color changes of the substrates were noticeable and complete within 5 min. Maximum color change of the OPD substrate occurred with 0.3 ml; however, adequate color change was noticed with 0.2 ml which was subsequently selected for assay development. This colorimetric change allowed visualization of membrane attached antibody

to a 1:10,000 dilution. Results with the 4C1N were disappointing. A purple color change was noted using 0.3 ml of the solution; however, this occurred with the membrane attached antibody to a 1:300 dilution. This experiment reflects that 1) the nitrocellulose paper was adequately binding protein (in the form of antibody), 2) the enzyme-substrate reaction was appropriate, and 3) the OPD appeared to be superior to 4C1N for ELISA development.

Bordetella pertussis: A human pool was titrated in the following manner. Two duplicate rows of cells were prepared in which 0.05 ml of a 1:10 dilution (approximately  $1.8 \times 10^8$  organisms/ml) of the Bordetella pertussis antigen was applied to each well with the exception of the first two. These wells received 0.05 ml of TBS and served as control wells for the experiment. All wells then received the blocking buffer and were rinsed with the wash buffer. A human pool was serially diluted from 1:10 to 1:10,000, and 0.1 ml of each dilution applied to a subsequent well. This step was followed by the addition of 0.1 ml of HPR anti-human serum. Each well was then rinsed with the wash buffer. A 0.2 ml aliquot of OPD was then added to each well. The control wells showed no color; however, a color change was evident in the antibody titration wells out to a 1:1000 dilution.

Titration of pertussis antigen. Two duplicate rows of cells were prepared in which 0.05 ml of serial dilutions ranging from a 1:10 dilution (containing approximately  $1.8 \times 10^8$  bacterial cells/ml) to a 1:5,000 dilution (containing approximately  $3.6 \times 10^5$  bacterial cells/ml) with exception of the first two wells. These wells received 0.05 ml of TBS

and served as control wells for the experiment. All wells the received the blocking buffer and were rinsed with the wash buffer. A .1 ml aliquot of a 1:1000 dilution of the human pool was added to each well. All wells were rinsed with the wash buffer. Each well then received 0.1 ml of HRP goat anti-human antibody diluted 1:3000. The wells were again rinsed with the wash buffer. All wells then received 0.2 ml of the OPD solution. Color changes were evident out to a 1:5000 dilution of the antigen preparation (approximately  $1.8 \times 10^5$  bacterial cells/ml or  $3.6 \times 10^4$  bacterial cells/0.05 ml). These results are summarized in Table 1.

Bordetella Antigen Dilution	*McFarland Units Applied	Dilution of Human Antiserum			
		1:10	1:100	1:1000	1:5000
1:10	0.02	**4+	2+	1+	0
1:50	0.001	4+	3+	1+	0
1:100	0.002	4+	4+	1+	0
1:500	0.0001	4+	2+	1+	0
1:1000	0.0002	3+	2+	1+	0
1:5000	0.00001	1+	1+	0	0
Control	0	0	0	0	0
<p>*1 McFarland unit is equivalent to approximately <math>0.9 \times 10^9</math> bacterial cells/ml.</p> <p>**Values are expressed as 0 (as comparable to control) to 4+ for comparison of the color intensity of OPD</p>					

Table 1. Bordetella pertussis antigen detection.

Legionella pneumophila antigen preparation: The antigen preparation was subjected to both the Biuret and Lowry protein detection procedures. The Biuret method showed no detectable protein; however, the results from the Lowry indicated that the antigen preparation concentration was approx-

imately .025 mg/ml. Subsequent calculations were determined from this estimate.

The legionella antigen preparation was titrated in a manner similar to Bordetella pertussis. Two duplicate rows of cells were prepared in which 0.05 ml of serial 5 and 10 fold dilutions of the preparation were applied. The first well of each row served as controls. All wells received the blocking buffer and were subsequently rinsed with the wash buffer. This step was followed by the addition of 0.05 ml of a 1:1000 dilution of rabbit legionella antiserum. Each well was then washed and inoculated with 0.2 ml of the OPD solution. Color changes were evident out to 250 pg/ml of the antigen preparation. Results from a typical assay are shown in Table 2.

Legionella Antigen Dilution	Protein Applied (ng/0.05 ml)	Dilution of legionella Antiserum				
		1:10	1:50	1:100	1:1000	1:5000
1:10	25.0	*4+	4+	4+	4+	3+
1:50	5.0	4+	4+	4+	2+	1+
1:100	2.5	3+	3+	2+	1+	1+
1:500	0.5	3+	3+	2+	1+	1+
1:1000	0.25	3+	3+	2+	1+	1+
*Values are expressed as 0 (as comparable to control) to 4+ for comparison of the color intensity of OPD.						

Table 2. Legionella pneumophila antigen detection.

## DISCUSSION

During the past decade, numerous immunoassays have gained wide acceptance as the methods of choice in the diagnosis of a number of disease states (10). The ideal considerations of a diagnostic test include speed, sensitivity, specificity, accuracy, safety, inexpensive reagents, potential for automation, long reagent shelf life, and broad applicability. Neither immunofluorescence or radioimmunoassay meet all these criteria. Many techniques have been developed recently for the immunological detection of antigens and/or antibodies. Enzyme immunoassays such as the ELISAs are among the most popular both in research (10) and clinical laboratory use for the diagnosis of bacteria, protozoans, and viruses as indicated in Tables 3, 4, and 5, respectively. In general, these tests are user-friendly, reliable, highly

<u>Chlamydia trachomatis</u>	<u>Streptococcus pyogenes</u>
Chlamydelisa (M.A. Bioproducts)	*TestPack (Abbott)
*Chlamydiazyme (Abbott)	*Ventrescreen (Ventrex)
	*Quest (Quidel Q)
<u>Mycoplasma pneumoniae</u>	*ICON (Hybritech)
Mycoplasmaelisa (M.A. Bioproducts)	
<u>Neisseria gonorrhea</u>	
*Gonozyne (Abbott)	
<u>Salmonella sp.</u>	
*(Kirkegaard and Perry)	

Table 3. Commercially Available Enzyme-Linked Immunosorbent Assays for Bacteria. Asterick (\*) denotes antigen detection.

sensitive and specific, and require little time to run. Additional considerations include that no power source or instruments are required for the performance of the tests, little equipment is required, and the reagents used are stable. Positive reactions are contrasted by outstanding color changes.

<u>Toxoplasma gondii</u>
Toxoelisa (M.A. Bioproducts)
Toxo-G (Abbott)
Toxo-M (Abbott)
Toxostat (M.A. Bioproducts)

Table 4. Commercially Available Enzyme-Linked Immunosorbent Assays for Protozoans.

The majority of these commercially available ELISA systems are designed to detect antibody levels in blood plasma or other biological fluids (i.e. urine) and few have been developed for the detection of microbial antigens. The Rotazyme (Abbott) and Pathfinder (Kallestad) kits which detect the presence of rotaviruses in stool specimens (Table 5); the Chlamydiazyme (Abbott), Gonozyne (Abbott), Salmonella detection kit (Kirkegaard and Perry), as well as the Test Pack (Abbott), Ventrescreen (Ventrex), Quest (Quidel Q), and Icon (Hybritech) for detection of Streptococcus pyogenes in throat swabs are designed for antigen detection. It appears advantageous to utilize ELISA systems directed to detect microbial antigens, particularly for the demonstration of their presence in certain body regions, biological fluids, or the external environment.

The commercially available ELISA systems were not designed to be



CYTOMEGALOVIRUS	ROTAVIRUS
Cytomegalisa (M.A. Bioproducts)	*Rotazyme (Abbott)
CMV-Stat (M.A. Bioproducts)	*Pathfinder (Kallestad)
Cytomegalisa M (Abbott)	
CMV total AB (Abbott)	RUBELLA
	Rubazyme (Abbott)
HTLV III	Rubazyme-M (Abbott)
(Abbott)	Rubelisa (M.A. Bioproducts)
(Electro-Nucleonics)	Rubelisa-M (M.A. Bioproducts)
(Ortho)	Rubestat (M.A. Bioproducts)
HEPATITIS-A ANTIGEN	RUBEOLA
Havab (Abbott)	Measelisa (M.A. Bioproducts)
Havab-M (Abbott)	
HEPATITIS-B ANTIGEN	VARICELLA
Ausab (Abbott)	Varicelisa (M.A. Bioproducts)
Auszyme II (Abbott)	
Corzyme (Abbott)	
Corzyme-M (Abbott)	
(Ortho)	
HEPATATIS-Be ANTIGEN	
HBe (Abbott)	
HEPATATIS-DELTA ANTIGEN	
Anti-Delta (Abbott)	
HERPES SIMPLEX	
Herpelisa 1 (M.A. Bioproducts)	
Herpelisa 2 (M.A. Bioproducts)	
MUMPS	
Mumpelisa (M.A. Bioproducts)	

Table 5. Commercially Available Enzyme-Linked Immunosorbent Assays for Viruses. Asterick (\*) denotes antigen detection.

utilized in microgravity, and thus, little concern was given to HMF requirements during their development. However, the Test Pack (Abbott) released in June, 1986 for purchase, has been tested in the NASA-JSC laboratory, and its technology appears to be promising for space station use. This system is solid phase utilizing an antigen capture filter

support, in which fluids are contained through diffusion into an internal absorptive sponge. The system requires approximately 10 minutes to run. The basic flow through system was successfully utilized in zero gravity experiments aboard the KC135.

The most common solid phase supports employed in ELISA systems have been polystyrene microtiter plates (16) and tubes (13) to which either antigen or antibody is passively adsorbed, although other supports such as polystyrene beads (8), sticks (3), and cuvettes (11) have been utilized. Antibodies and antigens have also been passively adsorbed to a number of other supports including polyvinyl (16), polycarbonate (14), aminoalkylsilyl glass (7), and silicone rubber (5). Covalent coupling of antigen or antibody to solid phase supports has been successful using cellulose (15), isothiocyanate (4), and polyacrylamide (15). Nitro-cellulose filter paper, used extensively in the development of DNA probe technology due to its ability to bind nucleic acids (1), has been found to nonspecifically bind proteins and has recently been employed as the binding surface on which immunoassays, such as the ELISA, are performed (9).

Results from the experiments conducted in this project and the existence of a commercial kit paralleling these findings, provide a current technology to be considered for the HMF. A major advantage to consider with the solid phase filter membrane systems is that the fluids involved in the system can be retained (i.e. little chance of spillage in the space craft environment). The use of solid phase filter supports will be soon expanding and kits will eventually be available

for the identification of those microorganisms, including viruses, expected to cause health problems in the space station environment. Since cell culture is usually required for the identification of viruses, this technology would certainly be an viable alternative.

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